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Pyridine Nucleotide Metabolism in *Escherichia coli*

IV. TURNOVER*

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SUMMARY

There are two pyridine nucleotide turnover pathways in *Escherichia coli*, the Gholson turnover cycle, with nicotinamide as an intermediate, and a second new pathway which does not involve nicotinamide. In exponentially growing cultures with a generation time of 60 min, the rates of these cycles are 39 and 102 molecules per s per cell, respectively.

We suggest that this second, heretofore undescribed turnover cycle proceeds with nicotinamide mononucleotide as the first intermediate, possibly formed by the DNA ligase-catalyzed cleavage of DPN.

An interesting aspect of pyridine nucleotide metabolism is its cyclic nature. Pyridine nucleotide turnover is believed to take place through a cycle proposed by Gholson (1) (see Fig. 1). A primary feature of the proposed cycle is that it involves the breakdown of DPN directly to nicotinamide (presumably through the action of an enzyme such as DPNase) (1). The rate at which this cycle operates in *Escherichia coli* has not been determined, although it has been shown to occur (2). In this paper, we report the rate of turnover of pyridine nucleotides through this cycle during exponential growth.

The pyridine nucleotide cycle as described by Gholson (1) may not be a complete description of the turnover of pyridine nucleotides within the bacterial cell. The discovery that DNA ligases of bacteria use DPN as an energy source in the synthesis of a DNA phosphodiester bond, resulting in the cleavage of the DPN molecule into AMP and NMN (3, 4) provides a route for DPN breakdown which is not taken into account by the cycle figured above. The results described here indicate that there are indeed at least two different and experimentally distinguishable pathways of pyridine nucleotide turnover.

EXPERIMENTAL PROCEDURES

^{14}C- and ^{3}H-labeled nicotinic acid and adenine were purchased from Amersham-Searle Corp. and New England Nuclear. Unless otherwise specified all biochemicals were obtained from Sigma. The [^{14}C]- and [^{3}H]nicotinic acid were purified before use as was described previously (5).

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DEAE-paper (Whatman DE 81) was purchased from H. Reeve Angel Co. and membrane filters (Bact-T-Flex nitrocellulose, type B-6) came from Schleicher and Schuell.

Bacterial Strains—The *E. coli* strains used in these experiments were a *nicC* strain, previously referred to as E126 and a *nicC* nicotinamide deamidase$^-$ strain (RS126). It has recently been suggested that the genes carried by these strains be referred to as *nadC pncA*$^+$ and *nadC pncA*, respectively (6). We will adapt this nomenclature and routinely refer to E126(*pncA*$^+$) or the nicotinamidase$^+$ strain and RS126(*pncA*) or the nicotinamidase$^-$ strain in the text. These strains were the generous gift of Dr. R. K. Gholson of Oklahoma State University.

The RS126(*pncA*) strain thus requires niacin for growth but cannot grow on nicotinamide as a substitute for niacin. This strain does not appear to be leaky since there is no measurable conversion of nicotinamide to any other compound by cells growing exponentially *in vivo*. Using comparable extracts of E126(*pncA*$^+$) and RS126(*pncA*), we find that at extract concentrations of the former sufficient to quantitatively convert nicotinamide to niacin, there is no detectable conversion by the RS126 extract.

Other Methods—Methods for growth of cultures, measuring uptake of radioactivity and the number of pyridine nucleotide molecules per cell, as well as for the determination of the rates of synthesis of DPN, TPN, and the rate of conversion of TPN to DPN have been described previously (5).

Growth of Cells in Differentially Labeled Niacin and Adenine—Cells were grown in 10 ml of an M9 glucose medium containing between 0.5 to 2 μg per ml of niacin per ml of ^{3}H- or ^{14}C-labeled for many generations. When the absorbance at 600 nm was between 0.5 and 1.0, ^{3}H- or ^{14}C-labeled adenine of high specific activity was added. The culture was allowed to grow for 1 or 2 hours in presence of radioactive adenine, and the cells were then filtered on a Millipore filter, washed with M9 medium, and transferred to 30 ml of a medium containing 1 μg per ml of unlabeled niacin. Samples (5 ml) were taken for chromatographic analysis at various times after the shift to the new medium. The increase in cell density, in optical density, and in the intracellular radioactivity were followed as described previously (5).

Chromatography of Pyridine Nucleotides—An aliquot (2 to 5 ml) of a culture was harvested by centrifugation at 7000 rpm in a Sorvall RC-2B centrifuge for 10 min and washed once with cold M9 medium (minus glucose). Cells were resuspended in a solution (0.5 ml) containing 0.01 M Tris, pH 7, 0.001 M EDTA, and 0.005 M β-mercaptoethanol, and were sonically disrupted

in a Raytheon sonic oscillator. The resulting mixture was acidified with 0.02 ml of cold 0.33 M HCl, and then centrifuged for 10 min at 7000 rpm. The supernatant was neutralized with 0.010 ml of 0.01 M Tris, pH 9.6, per 0.10 ml of supernatant. The sample (0.2 ml) was then immediately spotted on DEAE-paper with 0.05 ml of a solution containing 5 mg per ml of TPN, 5 mg per ml of DPN, and 10 mg per ml of nicotinamide as markers, and the chromatogram was developed with 0.25 M NH_4HCO_3 as solvent for 4 hours. The chromatogram was dried, the spots were visualized, and 1-cm strips were cut and counted on the ^3H and ^{14}C channels of a liquid scintillation counter.

RESULTS

Comparison of Niacin Metabolism in Different Strains of E. coli—Some of the rates of different reactions of niacin metabolism during exponential growth of strain 15T⁻ nic⁻ of *E. coli* were determined previously (5). For the experiments below, it was necessary to use a mutant of *E. coli* which is unable to convert nicotinamide to nicotinic acid: all studies described here used strain RS126(*pncA*) and the parent strain E126(*pncA*⁺) of *E. coli*. However, these strains grow somewhat more slowly than *E. coli* 15T⁻ nic⁻ under identical culture conditions (they exhibit a generation time of 60 min at 37° *versus* 40 min for 15T⁻ nic⁻). We have therefore redetermined all the constants of niacin metabolism for these strains which were previously determined for 15T⁻ nic⁻. The methodology used for these determinations was described in detail in the first paper of this series (5).

A comparison of the characteristics of niacin metabolism in the *pncA* strain (RS126), the *pncA*⁺ strain (E126), and 15T⁻ nic⁻ is shown in Table I. It is noteworthy that the TPN:DPN ratio is maintained at similar levels in all three strains, even though the generation times, the total pyridine nucleotide content per cell, and the rates of the different reactions vary between the three strains.

Loss of Niacin from pncA Strains—The pyridine nucleotide cycle (Fig. 1) predicts that the first reaction in DPN turnover is the degradation of DPN to nicotinamide (and ADP-ribose). In a bacterial strain which is unable to convert nicotinamide to nicotinic acid, such as RS126, nicotinamide should accumulate and eventually be excreted.

It was previously shown in 15T⁻ nic⁻ that intracellular niacin is totally conserved for many generations. If the turnover of pyridine nucleotides proceeded with nicotinamide as an intermediate, this should be manifested in the *pncA* mutant by an excretion of nicotinamide. In the experiment shown in Fig. 2, RS126(*pncA*) and E126(*pncA*⁺) were grown in a medium containing [^{14}C]niacin for many generations, and then shifted to an identical medium with unlabeled niacin. The intracellular radioactivity was totally conserved in the E126(*pncA*⁺) strain, but was lost exponentially in RS126(*pncA*). The half-life of the radioactivity in the mutant strain was 420 min.

The radioactivity excreted by the *pncA* strain during growth in the unlabeled medium was analyzed by paper chromatography and was shown to be exclusively nicotinamide. The rate of loss of radioactivity in the nicotinamidase⁻ strain is presumably a measure of pyridine nucleotide turnover with nicotinamide as an intermediate. These results indicate that a pyridine nucleotide molecule has a 50% probability of going through the Gholson pyridine nucleotide cycle every seven generations, under these conditions.

Loss of Adenine From DPN in RS126(pnc)A and E126(pncA⁺) Strains—The niacin moiety of the pyridine nucleotides is completely conserved in wild type *E. coli*. Thus, the pyridine ring of any pyridine nucleotide molecule which breaks down has a 100% probability of being recycled back to pyridine nucleotide, since niacin has no other metabolic fate. However, if a DPN or TPN molecule were broken down, the adenine ring would have a very low probability of returning to pyridine nucleotide, since adenine has a wide variety of metabolic fates, and the pyridine nucleotides contain a very minor fraction of the total adenine of the cell.

A second method of observing turnover was therefore employed by the use of a double label in the adenine and niacin moieties of the pyridine nucleotides. Cells were grown in a medium containing both radioactive adenine and niacin, and

TABLE I

Kinetic parameters of niacin metabolism of Escherichia coli strains

Reaction rates are given in units of molecules per s per cell. These parameters were all determined as described in detail by Lundquist and Olivera (5). *E. coli* 15T⁻ nic⁻ was grown in an M9-glucose medium supplemented with 4 μg per ml of thymine, 380 μg per ml of arginine, 300 μg per ml of methionine, 140 μg per ml of tryptophan, and 1 μg per ml of niacin; E126 and RS126 were grown in M9-glucose medium supplemented with 1 μg per ml of niacin. The addition of the other supplements to cultures of RS126 and E126 did not significantly change the generation time.

Parameters	15T⁻ nic⁻	*pncA*⁺ (RS126)	*pncA* (E126)
Generation time (min)	40	60	60
Total pyridine nucleotide molecules per cell	1.90×10^6	1.69×10^6	1.42×10^6
R_u, actual rate of DPN synthesis	548	323	308
R_1, actual rate of TPN synthesis	266	206	146[a]
R_2, rate of TPN breakdown	140	131	89[a]
Net DPN synthesis	422	248	212
Net TPN synthesis	126	75	57[a]

[a] Calculated assuming that breakdown to nicotinamide occurs exclusively from DPN, and not from TPN.

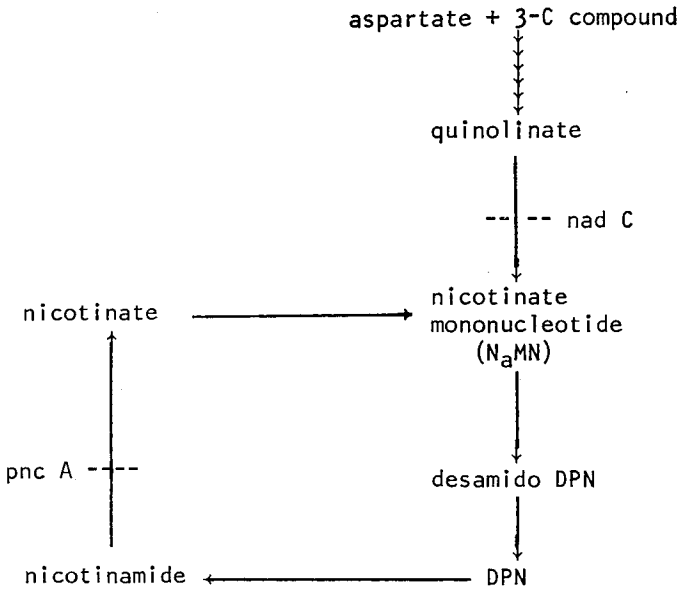

FIG. 1. The pyridine nucleotide cycle as proposed by Gholson (1).

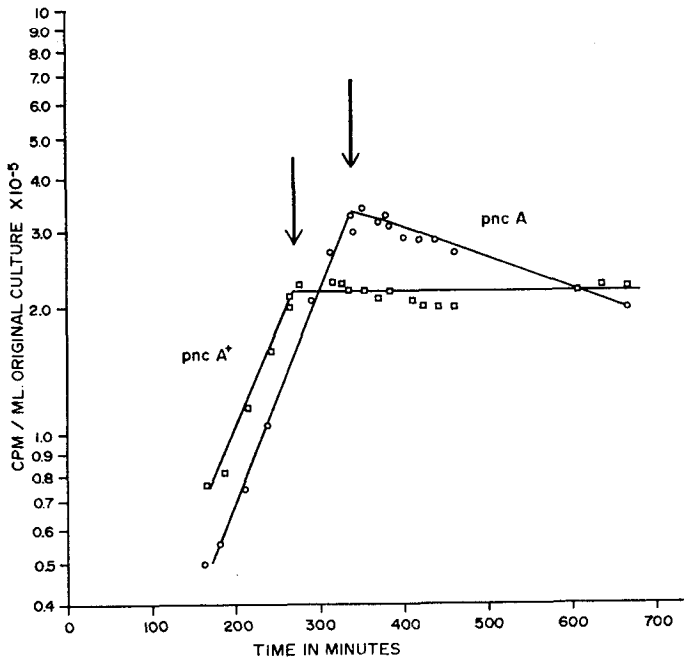

FIG. 2. Loss of niacin compounds in RS126(*pncA*) strain during exponential growth. Cells were grown exponentially in an M9-glucose medium containing 1 μg per ml of [^{14}C]niacin (59 Ci per mole). At the times indicated by *arrows* the cultures were filtered on a Millipore filter and resuspended in a 10-fold (for E126(*pncA*$^+$)) or 20-fold (for RS126(*pncA*)) greater volume of M9-glucose containing 1 mg per ml of nonradioactive niacin. The intracellular radioactivity was measured by filtering aliquots of the culture on a Millipore filter and measuring the radioactivity retained.

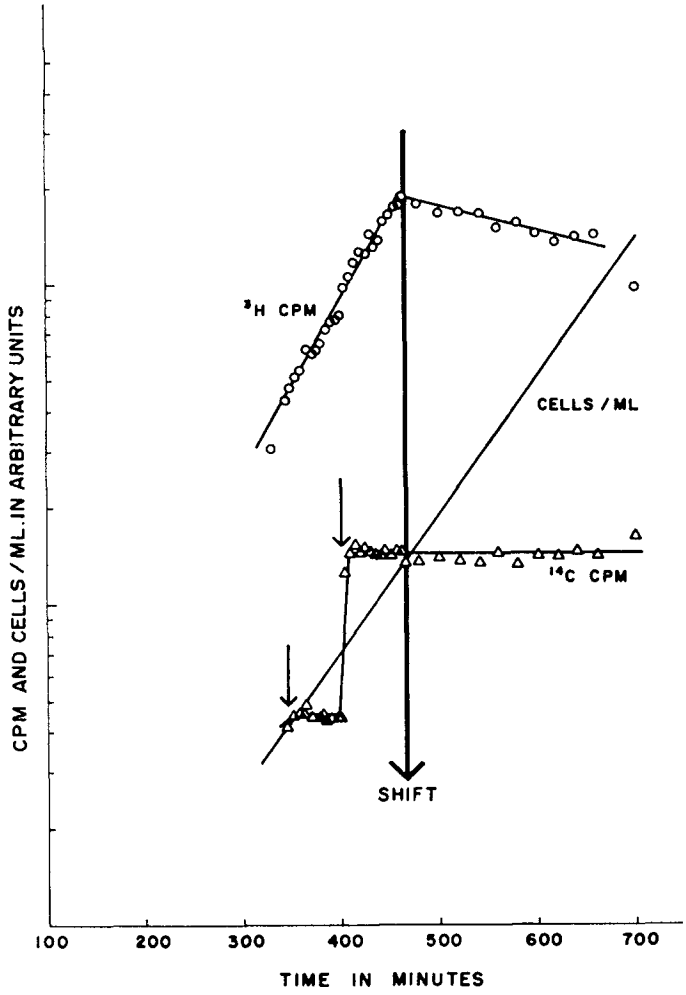

FIG. 3. The growth of the nicotinamidase$^-$ strain in the presence of [^{3}H]niacin and [^{14}C]adenine. A culture of *Escherichia coli pncA* was grown in an M9 medium containing [^{3}H]niacin (0.55 μg per ml, 787 Ci per mole) for many generations. At the times indicated by *arrows*, [^{14}C]adenine was added (0.31 μg per ml of culture 120 min before the shift, 0.62 μg per ml of culture 60 min before the shift, specific activity = 231 Ci per mole). At the time indicated the culture was filtered on Millipore and resuspended in a 6-fold greater volume of unlabeled M9 medium containing 1 μg per ml of niacin. The uptake of radioactivity was followed as described under "Experimental Procedures," while the number of cells per ml of the original culture was calculated using a Coulter counter and making corrections for the dilution of the culture after the shift. At the time of the shift the uptake of niacin (^3H) was 1.85×10^5 cpm per ml, and of adenine (^{14}C) 1.30×10^5 cpm per ml with a cell density of 7.1×10^8 cells per ml.

then shifted to a medium which had no radioactivity. In E126(*pncA*$^+$) we expect that every time any of the chemical bonds linking the niacin ring to the adenine ring in a pyridine nucleotide molecule is broken down, it would result in loss of the adenine ring from the pyridine nucleotide pool. Since the niacin ring recycles back to the pyridine nucleotide pool with 100% efficiency a preferential loss of label in the adenine moiety should be observed. However, if pyridine nucleotide turnover proceeded with breakdown to nicotinamide as an obligatory step, then in the nicotinamidase$^-$ mutant, every time a pyridine nucleotide molecule were broken down, not only would the adenine be lost to the pyridine nucleotide pool, but the pyridine ring should be lost as well, since this strain is incapable of recycling any nicotinamide back to pyridine nucleotide. If these conditions were true, then in RS126(*pncA*), the ratio of radioactivity in adenine to radioactivity in niacin in the pyridine nucleotides should not change after the cells are transferred from a labeled to an unlabeled medium (since there is a proportional loss of both labels at all times).

These experiments are shown in Figs. 3, 4, and 5. In the experiment in Figs. 3 and 4 the cells were grown in medium containing [^{3}H]niacin and [^{14}C]adenine. After the culture was shifted to the unlabeled medium, an increase in the ^{3}H:^{14}C ratio was observed in DPN (Fig. 4). As a control (Fig. 5), the cells were also grown in a medium containing [^{14}C]niacin and [^{3}H]-adenine. After the shift, a *decrease* in the ^{3}H:^{14}C ratio was observed in the DPN peak. Fig. 6 shows the data from these and similar experiments plotted on a normalized scale: the half-life of the adenine moiety in a DPN molecule ($t_{1/2}$) for E126(*pncA*$^+$) under these conditions is 140 min. The preferential loss of radioactive adenine (compared to niacin) from the pyridine nucleotide pool was observed both in the wild type strain ($t_{1/2}$ = 140 min) and in the mutant which is unable to convert nicotinamide to niacin ($t_{1/2}$ = 160 min). Thus, if a DPN or TPN molecule breaks down, the niacin moiety has a much higher probability of returning to the pool than the adenine moiety in both the nicotinamidase$^-$ and the nicotinamidase$^+$ strains, despite the fact that the nicotinamidase$^-$ strain is unable to recycle nicotinamide.

DISCUSSION

Our results indicate that mutants of *E. coli* which are incapable of converting nicotinamide to nicotinic acid continuously

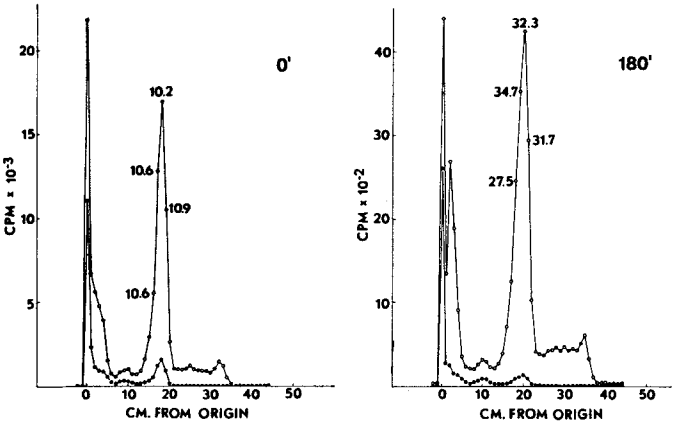

FIG. 4. Chromatography of extracts of cells grown in [^{3}H]-niacin and [^{14}C]adenine. Cells were grown as described in the legend to Fig. 3. An aliquot of cells (5 ml) was harvested immediately after resuspension in the unlabeled medium, and again after growth for 3 hours in the unlabeled medium. The cells were subjected to chromatography and the resulting chromatogram was analyzed for radioactivity as described under "Experimental Procedures." The major peak which moves from the origin is the DPN peak. The *open circles* represent the radioactivity in the ^{3}H channel, while the *closed circles* represent the radioactivity in the ^{14}C channel. The radioactivity in the ^{3}H channel at the origin is due to spillover from the ^{14}C channel. The numbers beside each fraction along the DPN peak are the ^{3}H:^{14}C ratio for that particular fraction. The purity of this peak was confirmed by extracting some material from this region of the chromatogram and reducing the DPN by the alcohol dehydrogenase reaction. It was found that after reduction the radioactivity traveled with authentic DPNH on rechromatography, with the same ratio of ^{3}H:^{14}C.

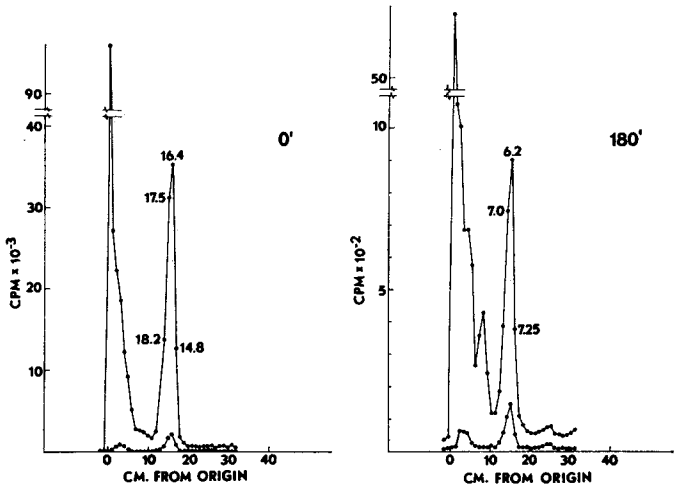

FIG. 5. Chromatography of extracts of RS126 cells grown in [^{3}H]adenine and [^{14}C]niacin. The RS126(*pncA*) strain of *Escherichia coli* was grown for many generations in an M9 medium containing 0.57 μg per ml of [^{14}C]niacin (8.7 Ci per mole). When the absorbance at 600 nm was approximately 1.0, [^{3}H]adenine was added (4 μg per ml of culture, 1100 Ci per mole). One hour later, the cells were filtered on a Bact-T-Flex B6 Millipore filter and resuspended in an unlabeled M9 salts medium containing 1 μg per ml of niacin. Cells were then harvested and subjected to chromatography as described in the legend to Fig. 4. The numbers along the DPN peak represent the ^{3}H:^{14}C ratios for those fractions.

excrete nicotinamide. The half-life of the niacin ring in cells of this mutant is 420 min. From this half-life, the rate of breakdown to nicotinamide, R, can be calculated. It can readily be shown that:

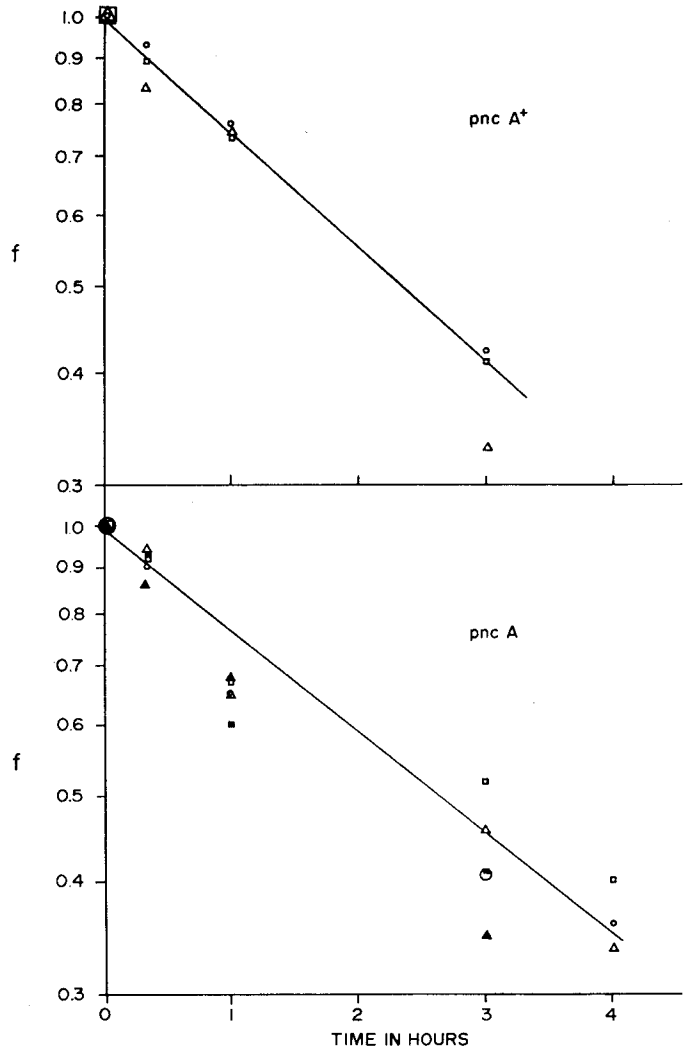

FIG. 6. A logarithmic plot of f, the fraction of radioactivity in adenine remaining in the pyridine nucleotide pool as a function of time of growth after shifting to the unlabeled medium. The experimental points are derived from experiments similar to those shown in Figs. 3, 4, and 5. f is equal to (radioactivity in adenine/radioactivity in niacin)$_t$/(radioactivity in adenine/radioactivity in niacin)$_{t=0}$, where t is the time. The different symbols are calculations for different fractions along the DPN peak after chromatography. *Open symbols* refer to experiments in which adenine was labeled with ^{3}H and niacin was labeled with ^{14}C, while *closed symbols* refer to experiments in which the labeling was reversed. The *top graph* shows the experimental results using the E126(*pncA*$^+$) strain, the *bottom plot* is for the RS126(*pncA*) mutant.

$$R = \frac{0.693}{t_{1/2}} \times [\text{No. pyridine nucleotide molecules/cell}]$$

$$= \frac{(0.693) \times (1.42 \times 10^6 \text{ molecules/cell})}{(420 \times 60) \text{ s}}$$

$$= 39 \text{ molecules/s/cell}$$

This rate of breakdown of pyridine nucleotide to nicotinamide in RS126(*pncA*) should be equal to the rate of DPN turnover through the Gholson pathway in E126(*pncA*$^+$) strain under these growth conditions.

The double label pulse-chase experiments described above indicate that in the RS126(*pncA*), which is unable to recycle nicotinamide, there is nevertheless a preferential loss of adenine

compared to niacin from the pyridine nucleotide pool. We interpret this to mean that there is a pathway of pyridine nucleotide breakdown and turnover which does *not* involve nicotinamide. Indeed, it would appear that this is the major pathway of pyridine nucleotide turnover since the rate of disappearance of adenine from the pyridine nucleotide pool (half-life = 140 min) is more rapid than the rate of excretion of the pyridine ring in the nicotinamidase$^-$ mutant (half-life = 420 min).

We may estimate the rate of turnover of pyridine nucleotides through the pathway not involving nicotinamide. Since it is experimentally difficult to determine double label ratios in TPN, we will assume for the purposes of this calculation that (*a*) the rate of interconversion of DPN and TPN is reasonably rapid compared to the rate of pyridine nucleotide turnover, and thus, that the relative rate of decay of the adenine label is roughly the same in DPN or TPN, and (*b*) the probability of labeled adenine returning to the pyridine nucleotide pool during a chase is sufficiently small so it can be neglected.

In the nicotinamidase$^+$ strain (E126), we can therefore calculate the total rate of turnover, $Rpnc_1 + Rpnc_2$ (where $Rpnc_1$ is the rate of turnover through the Gholson cycle and $Rpnc_2$ is the rate of turnover through all pathways not involving nicotinamide). This is determined by the expression:

$$Rpnc_1 + Rpnc_2 = \frac{0.693}{t_{1/2}} \text{[pyridine nucleotide molecules/cell]}$$

$$= \frac{0.693}{(140 \times 10)} (1.7 \times 10^6)$$

$$= 140 \text{ molecules/s/cell.}$$

Since $Rpnc_1$ = 39 molecules per s per cell then $Rpnc_2$ = 101 molecules per s per cell.

An independent calculation of $Rpnc_2$ may be made from the data for the nicotinamidase$^-$ strain (RS126). In this mutant, all the preferential loss of adenine should come from $Rpnc_2$, since the strain is unable to successfully complete the Gholson cycle. Thus,

$$Rpnc_2 = \frac{0.693}{t_{1/2}} \text{[pyridine nucleotide molecules/cell]}$$

Since in this strain $t_{1/2}$ = 160 min, and there are 1.42×10^6 pyridine nucleotide molecules per cell, then:

$$Rpnc_2 = \frac{0.693}{(160)} (60) (1.42 \times 10^6) = 103 \text{ molecules/s/cell}$$

Thus, the rate of the second turnover cycle, $Rpnc_2$, is over 2 times the rate of the first pyridine nucleotide cycle involving nicotinamide ($Rpnc_1$).

We might speculate briefly on the biochemical nature of this turnover cycle which does not involve nicotinamide. In the Gholson cycle, the conversion of nicotinamide to niacin by nicotinamidase is the point at which deamidation takes place. This implies that this new pathway either does not involve deamidation, or that it involves deamidation of the nicotinamide ring at a level other than the free base. We have examined extracts of *E. coli* strain RS126 for the ability to deamidate compounds containing nicotinamide (other than the free base itself), and we have detected an activity which deamidates nicotinamide mononucleotide.[1] A similar activity was first described by Fyfe and Friedmann (7) from *Clostridium sticklandii* and has also been shown in *Propionibacterium shermanii* (8, 9). It therefore seems likely that this NMN deamidase is fairly widely distributed in bacteria. With this enzyme, it becomes possible to define a new pyridine cycle on the basis of enzymatic activities which have all been detected in *E. coli* extracts:

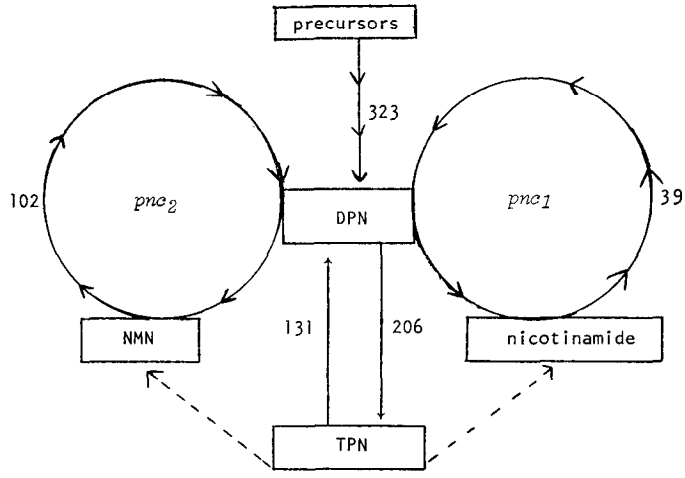

FIG. 7. A hypothetical scheme for the flow of the niacin ring in *Escherichia coli* E126(*pncA*$^+$) growing exponentially. The flow chart of the pyridine ring is shown very schematically. Numbers represent the average rate of the flow pathways in molecules per s per cell. The rate for TPN → DPN (131 molecules per s per cell) would include all breakdown of TPN, including degradation to NMN and nicotinamide (shown as *dotted arrows*). It is assumed in the representation that all of the second turnover cycle proceeds through NMN as an intermediate.

$$\text{DPN} \rightarrow \text{NMN} \rightarrow \text{N}_a\text{MN} \rightarrow \text{desamido-DPN} \rightarrow \text{DPN}$$

The first reaction may be catalyzed by DNA ligase (3, 4) and the second step by NMN deamidase. The other reactions are the standard reactions of the Preiss-Handler pathway (10).

A second possibility is that this new turnover pathway does not involve any deamidation at all. If there were breakdown of DPN to NMN, the NMN could conceivably be directly converted to DPN by desamido-DPN pyrophosphorylase. It has been shown that this enzyme has a strong, but not absolute preference for N_aMN over NMN; the residual activity for the amide could account for the turnover observed (11).

The possible involvement of DNA ligase in this turnover cycle is of considerable interest. This involvement is supported by our recent finding that in a temperature-sensitive DNA ligase mutant of *E. coli*, DPN turnover is much reduced at the nonpermissive temperature although it appears to be normal at the permissive temperature.[2] Experiments are in progress to measure quantitatively the role which DNA ligase plays in DPN turnover under different physiological conditions.

These studies, coupled with our previous results, lead to a definition of the pathways through which the niacin ring flows in the bacterial cell: the synthetic pathway from endogenous or exogenous precursors to DPN, the synthesis of TPN, the breakdown of TPN, and the two turnover cycles. Although the biochemical details of some of the pathways remain to be elucidated, the rates for the major routes for synthesis, breakdown, and recycling of all compounds containing the niacin ring can be determined. A summary flow chart is shown in Fig. 7.

Note Added in Proof—A recent publication (by Andreoli *et al.*) has suggested an alternative scheme to the turnover cycle given in Fig. 1 of this paper. Dr. A. J. Andreoli has also kindly pointed

[1] S. Sirilan and B. Olivera, unpublished results.

[2] P. Manlapaz-Fernandez and B. Olivera, unpublished results.

out to us cross-feeding experiments which demonstrate a pyridine nucleotide cycle in *Escherichia coli* (13). NMN deamidase has been purified and studied in more detail since this paper was submitted (14).

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